Investigating the Modulation Effects of Black Raspberry Polyphenols on Gut Microbial Metabolome

Research Thesis

Presented in Partial Fulfillment of the Requirements for Graduation with Research Distinction in Microbiology in the undergraduate colleges of The Ohio State University

By

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Abstract

The gut microbiome affects many aspects of human health including response to cancer treatments. A healthy gut microbiota serves important functions in maintaining the gut barrier's structural integrity and enhance responses to certain cancer treatments. Recent work has demonstrated that certain gut bacteria can modulate the host response to immune checkpoint inhibitors (ICIs), and, in mice, response to ICIs can be increased by increasing the abundance of probiotics like *Akkermansia muciniphila, or Lactobacillus acidophilus*. Diet-based interventions hold promise for translating microbiome modification into a clinical setting; however, progress has unfortunately been slowed by a lack of evidence regarding how specific food products can affect microbes, particularly in complex communities such as the gut microbiome. Increasing the abundance of probiotics thus represents a promising approach to modulate the likelihood of response to ICIs in humans. As such, it is important to investigate how certain important bacterial species can be enriched in gut microbiota via the use of polyphenol-rich fruits. The objective of this project focuses on the use of a novel *in vitro* human colonic model to simulate a colon environment without the interference from the host, to study the interaction between gut microbes from study participants of a lung cancer prevention trial and polyphenol-rich black raspberry (BRB) extract; and to characterize the microbial metabolites. After analyzing representative HCM samples using Liquid-chromatography Mass spectrometry (LC/MS) and statistical analysis, all three sections of the colon had shown distinct metabolic profiles for endogenous polar metabolites during BRB treatment compared to pre-treatment; two phenolic compounds were statistically significant in ascending and

transverse colon when compared between treatment phase versus post-treatment phase. Meanwhile, six short-chain fatty acids (SCFAs) were detected with statistical significance in ascending colon, and two SCFAs were detected with statistical significance in descending colon during the BRB treatment. In summary, this study indicated that BRB extract is capable of remodeling gut microbial metabolism and alter the levels of many potentially important microbial metabolites.

Acknowledgments

I would like to thank the Zhu lab for their great support in this difficult but fruitful scientific journey, especially to my mentor Dr. Chris Zhu, for providing a bird's eye view on the topic whenever I am in doubt and teaching me to be a critical thinker. Without his support, it will be impossible for me to complete the project as planned. I would like to thank Dr. Kwiek, for his help throughout my days as a Microbiology major student and for serving as my committee member. I would like to express my sincere gratitude to Ms. Shiqi Zhang for her constant help and training when I first enter the lab till I carrying out my experiment; the same gratitude goes to Mr. Fouad Choueiry for his support of my experiment by ensuring the supply of HCM and coaching me on proposal writing. I wish them all the best for their upcoming candidacy exam. I would also like to thank Dr. Li Chen, for her thorough training on mass spectrometry and detailed supervision whenever I am in the laboratory, without those, I would have repeated the same experiment many times. In addition, I would like to thank Undergraduate Research Office for awarding me the Undergraduate Research Scholarship. Lastly, I would like to thank all my friends, who had to lend me a listening ear when I had an emotional breakdown during this process and for being my cheerleaders when I was down. I would also like to thank myself, for not giving up and pressed on when facing unprecedented challenges. It was a special experience to complete a project during a pandemic and such fond memory will accompany me through the ups and downs in life in the future.

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Introduction

There are thousands of different types of microbes living in and outside of the human body. Out of many habitats, one particularly important place where trillions of microbes exist in the human gut, which houses many microbes in its mucous membrane and could take up 1-3% of our weight. In a healthy human, most bacteria can be divided into five phyla (*Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria,* and *Verrucomicrobia*) and with a majority of them being commensal bacteria [1]. Many studies involving animal models and clinical analysis had shown that these microbes play an important role in regulating homeostasis, metabolic processes and preventing gut inflammations, thus, creating a symbiosis relationship [2]. It is well-known that the delicate balance of microbiota in the gut is involved in maintaining gut integrity, and gut microbiota can be influenced by individual diets [3][4]. When the gut barrier is compromised, it provides an opportunity for pathogenic invasion. Thus, altering the delicate microbiome balance contributes to the inflammatory response [5]. As diet is believed to be a major factor in influencing this microbial modulated immune response, fluctuations in nutrient intake may instigate a vicious cycle, eventually leading to inflammatory bowel disease (IBD) such as ulcerative colitis, where ulcers form in the colon. This is a well-known precursor to colorectal cancer formation [6].

In addition, recent work has demonstrated that certain gut bacteria modulate response to immune checkpoint inhibitors (ICIs), and, in mice, response to ICIs can be increased by increasing the abundance of probiotics such *as Akkermansia mucinphilia* [7][8]. Diet-based interventions hold promise for translating microbiome modification into

a clinical setting; however, progress has unfortunately been slowed due to a lack of evidence regarding the ways in which specific food products can affect microbes, particularly in complex communities such as the gut. Increasing the abundance of probiotics via nutritional intervention thus represents a promising approach to modulate the likelihood of response to ICIs in humans. As such, it is important to maintain a healthy gut microbiota and correct any dysbiosis as it serves important functions in maintaining the gut barrier's structural integrity and enhance responses to certain cancer treatments [9][10].

In recent years, there are numerous attempts in the scientific community to characterize how specifically can diet influence microbial diversity and how such change can influence human health. Fruit rich in polyphenol have captured much attention as polyphenol has antimicrobial, anti-cancer and anti-inflammatory properties [11]. Like many other berries, such as strawberry and pomegranate, black raspberry (BRB) contains a rich source of phenolic compounds like anthocyanins, ellagitannins or quercetin [12]. In an animal study done on F344 rats, ellagic acid, a phenolic acid found in BRB, can prevent carcinogenic transformation [13]. Other compounds, such as β-sitosterol or ferulic acid, were able to curb premalignant and malignant formation for various forms of aerodigestive cancers [14][15]. Polyphenols can be dived into flavonoids or non- flavonoids and BRB contains both groups [16]. It is also reported that fruits rich in polyphenols can promote the growth of probiotics, restore dysbiosis, regulate host body weight and thus improve the host's overall health [17][18][19]. Many of these studies were done in animal models or healthy volunteers, which has important scientific significance with a real-life simulation of how diet, microbiota and host will interact. Yet, the limitation of such studies includes

restriction in the frequency of sample collection, unable to control all parameters involved and unable to establish a clear link of how diet and microbiota interact. All these limitations, however, can be overcome by the use of *in vitro* models such as the Human Colonic Model (HCM) established in our lab. Therefore, in our study, we plan to utilize a short-term (2-weeks) intervention study in HCM to simulate the growth environment for gut microbes while removing host responses to specifically focus on the interaction between polyphenol and gut microbiota from participants of a lung cancer prevention trial.

We hypothesize that the dietary intervention of BRB will remodel gut microbial metabolism and alter the production of potentially beneficial/anti-inflammatory metabolites. The short-term goal of our project aims to uncover how black raspberry extract can influence human gut microbial metabolism and how these metabolites could modulate host health in cancer prevention. In the long run, we aim to elucidate the mechanism of the interplay among dietary intervention, gut microbiota and microbial metabolism and cancer, so that eventually, we can apply the knowledge in the clinical setting of cancer prevent/treatment.

Materials and Methods

Chemicals

HPLC-MS-grade acetonitrile, ammonium acetate, formic acid, and acetic acid were purchased from Fisher Scientific (Pittsburgh, PA, USA). Phenolic compound standards including protocatechuic acid, 3,4-Dihydroxyphenyl ethanol, 3,4-dihydroxyphenylacetic acid, gallic acid, caffeic acid, isoferulic acid, urolithin A, transresveratrol, equol, daidzein, homovanillic acid sulfate, genistein, naringenin, phloretin, kaempferol, epicatechin, catechin, enterolactone, quercetin, hesperetin, epigallocatechin, isorhamnetin, myricetin, chlorogenic acid, roseoflavin, phlorizin, epicatechin gallate, epigallocatechin gallate, quercetin 3-D-galacoside, and rutin were purchased from Cayman company (Ann Arbor, MI, USA). 10mM volatile free acid mix, including acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, hexanoic acid, isocaproic acid, n-heptanoic acid and 2-methylbutyric acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). $^{13}C_4$-sodium butyrate was purchased from Avanti polar lipids, Inc. (Alabaster, AL, USA). Pyridine, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC·HCl) and 3-Nitrophenylhydrazine hydrochloride (3NPH·HCl) were also purchased from Sigma-Aldrich.

Human Colon Microbiota

Fresh fecal samples were obtained from two study participants of a lung cancer prevention trial. Approval was obtained from the Institute Review Board (IRB) committee of the Ohio State University before the study (clinical trial approval number NCT04267874), and informed consent was obtained from the fecal donors.

Microbiome Extraction

Microbiome from study participants of a lung cancer prevention trial was obtained using fresh stool sample by mixing with phosphate-buffered saline and vortex. 1 ml of the resultant solution was added to 9ml of Gifu Anaerobic Broth (GAM, HIMEDIA) and cultured overnight [20]. All above process was done in the anaerobic chamber to preserve obligate anaerobes (Coy Lab, Grass Lake, MI, USA). 3 ml of the microbiome culture was added into each vessel representing different sections of the colon.

Human Colonic Model Set-up

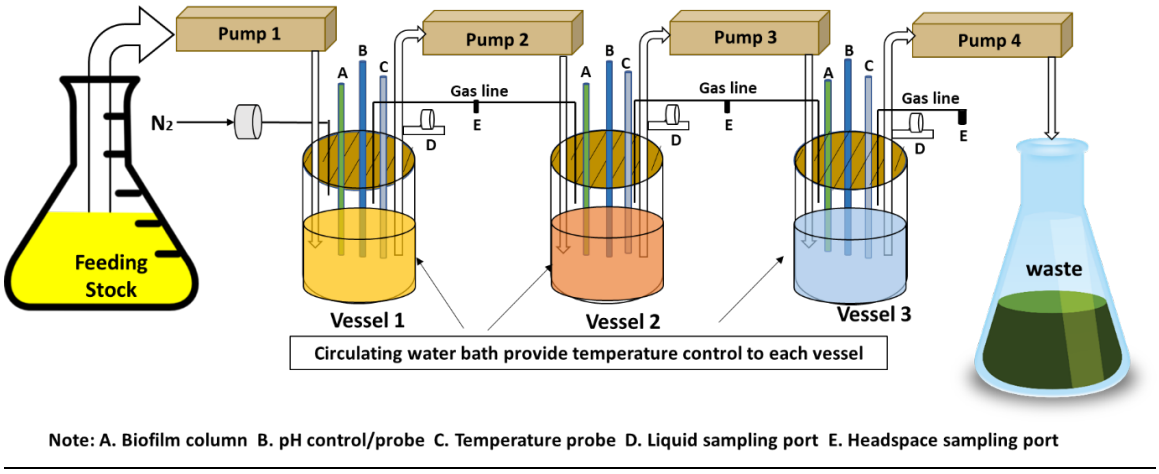

Figure 1. A schematic of Human Colonic Model (HCM), which consist of three pH and temperature controlled bioreactors to represent three segments of the large intestine, feeding bottle representing food content from small intestine (medium pre-treated with stomach enzyme and incubated for 2 hours to mimic the digestion, two bottles containing acid and base respectively to control the pH and a waste vessel to represent excretion.

The Human Colonic Model (HCM) was set up using consist of three bioreactors to simulate segments of the large intestine in humans (Figure 1). Vessel 1, 2 and 3 represent

the ascending, transverse and descending colons, respectively. Vessels contain various volumes of medium respectively as we described earlier [21]. The vessels were maintained at 37 °C by a water bath to simulate body temperature while the anaerobic environment was maintained by constant N_2 gas flushed through the entire system. Also, 3 ml of the recovered gut bacteria were inoculated into each vessel. 100 ml of medium was transferred three times a day with automated pumps controlled by in-house software and electric controllers to simulate human food consumption. The HCM model had run for a total of 6 weeks, weeks 1-2 as equilibrium phase, weeks 3-4 for treatment followed by 2 weeks post-treatment. The system was monitored with daily plate culture as described below.

HCM Feeding Medium

The feeding medium was prepared using the in-lab protocol. Briefly, 59.5g of Gifu Anaerobic Broth (GAM, HIMEDIA) was added to 4L of distilled water, hydrochloric acid was added to adjust to pH 2. The mixture was autoclaved for 30 minutes then cooled to 37℃ and shake in an incubator for an hour. 2.4g/L of bile salt and 0.4g/L pancreatin were added to the mixture inside the biosafety hood, sodium hydroxide was added to bring pH to 5.5. The mixture was shaken in an incubator for 2 hours at 37℃. For black raspberry treated media, 5ml/L of concentrated black raspberry extract supplement (Berri Product LLC) was added to the mixture.

Daily Culture Plate

Daily culture plating was performed to monitor the overall microbial growth in the HCM system and ensure the stability of the HCM. 1 ml of liquid extracted from each vessel every day and the sample was brought into the anaerobic chamber for culture. Samples from three vessels undergo 6 fold serial dilution before culture on a Gifu Anaerobic Broth

agar plate. The agar plate was left in a 37℃ incubator for 24 hours and the colonies were counted. Additional samples were collected and stored in -80℃ at the same time for additional metabolite and genomic analysis.

Sample Preparation and Liquid-Chromatography Mass Spectrometry (LC/MS)

All liquid chromatography-mass spectrometry (LC/MS) related experiments were performed on a Vanquish UHPLC system coupled with a hybrid Quadrupole Orbitrap Q Exactive™ mass spectrometer system (Thermo Electronics, Waltham, MA). Briefly, the UHPLC system has a temperature-controlled autosampler for high-throughput analysis, a column compartment and a binary pump for separation of analytes, the hybrid Quadrupole Orbitrap Q Exactive™ mass spectrometer can achieve high-resolution analysis (up to 140,000) for accurate m/z measurement and therefore enable confident compound identification.

Polar Metabolite Extraction

Both intracellular and extracellular polar metabolites extraction were performed [20]. Briefly, 800 µl of thawed HCM samples were centrifuged at 1400 rpm for 10 minutes, supernatants were transferred to sterile Eppendorf tubes. For extracellular metabolite extraction, 100 µl of the supernatants were transferred to new sterile Eppendorf tubes and 250 µl of methanol was added to each tubes containing supernatants. For intracellular extraction, the pellets from the first round of centrifugation were washed with 500 µl PBS and centrifuged again at 14000 rpm for 10 minutes, supernatant from the tube was removed and the whole process was repeated three times, then 250 µl of methanol was added to the pellets. The above step was performed on the supernatant containing exogenous metabolites too. The following steps described below were performed on both endogenous

and exogenous samples. The samples were vortex for 2 minutes before they were put in -20℃ for 20 minutes. Then, the samples were centrifuged again at 14000 rpm for 20 minutes, and the resultant supernatants were transferred to a clean LC valve for analysis. The samples were tested twice in both positive and negative mode. Each time, 10 µl aliquot was injected into the HPLC-MS/MS instrument for analysis using Xbridge BEH Amide (2.5um, 2.1x150mm, Waters, Milford, MA, USA) at 0.350 ml/min using mobile phase A (5 mM ammonium acetate in 10% acetonitrile/ 90% water and 0.2% acetic acid), mobile phase B (5 mM ammonium acetate in 10% acetonitrile/ 90% water and 0.2% acetic acid).

Phenolic Metabolite Extraction

Samples were prepared using a protocol established by a previous study [22], 150 µl of HCM samples were dissolved in 1.5 ml of 80% methanol. The samples were sonicated for half an hour at room temperature and centrifugation at 1000G for 10 minutes at 4℃. The supernatant collected using a 0.2 µm PTFE filter. 500 µl was transferred to a clean LC valve and loaded onto the instrument. The samples were tested twice in both positive and negative mode. Each time, 10 µl aliquot was injected into the HPLC-MS/MS instrument for analysis using XTERRA RP 18 (3.5um, 3.9x100mm, Waters, Milford, MA,USA) at 0.300 ml/min using mobile phase A (89.9% water/ 10% acetonitrile/0.1% formic acid), mobile phase B (69.9% water/ 30% acetonitrile/ 0.1% formic acid).

Short-Chain Fatty Acid Extraction

Samples were prepared using methods described by Chen *et al* [23]. A range of short-chain fatty acids (SCFA) standards were prepared using 10mM volatile free acid mix (Sigma–Aldrich, St. Louis, MO, USA) as described in Table 1:

Table 1: table showing standard preparation of SCFA from 10mM acid mix for the calibration curve

Dilution factor	acid mix/ µl	2-methyl butyric acid/ µl	50% acetonitrile/ µl
2.5x	80.00	80.00	40.00
5.0x	40.00	40.00	120.00
7.5x	26.67	26.67	146.66
10.0x	20.00	20.00	160.00

After which, 20 µl of each 1mM 13C4 sodium butyrate, 200mM 3-nitrophenylhydrazine hydrochloride solution and 120 mM M-(3-dimethylaminopropyl)-N'-ethylcabodilmide-6% pyridine solution were added to the standards respectively. The standards above were then diluted by 10 times, 100 times and 1000 times and result in 16 samples in total, serving as a calibration curve to quantify the amount of SCFA present in the HCM samples. The concentration of SCFAs was calculated and the peak area was used to draw a calibration curve to quantify the amount of SCFAs present in HCM samples.

For HCM samples, 1 ml were transferred into a fresh Eppendorf tube and centrifuge for 10 minutes at 14000 rpm. 20 µl of the supernatant was transferred to a new Eppendorf tube, 20 µl of each 1mM $^{13}C_4$ sodium butyrate, 200mM 3-nitrophenylhydrazine hydrochloride solution and 120 mM M-(3-dimethylaminopropyl)-N'-ethylcabodilmide-6% pyridine solution was added to the samples respectively. Both standard and HCM samples

were vortex before putting into a 40℃ water bath for half an hour. Then, they were cooled on ice for 1 minute and diluted with 1.92 ml of 10% aqueous acetonitrile. The prepared samples were then diluted 20 times to fit the range of the standards, 500 µl was transferred to a glass valve and loaded onto the instrument. Vanquish UHPLC System (ThermoFisher, Waltham, MA, USA) was used for metabolites analysis, 10 µl aliquot was injected into the HPLC-MS/MS instrument for analysis using Acquity UPLC CSH C18 Column (1.7um, 2.1x100mm, Water, Milford, MA, USA) at 0.500 ml/min using mobile phase A (100% water/ 0.01% formic acid) and mobile phase B (100% Acetonitrile/ 0.01% formic acid).

Statistical analysis

The SCFA spectral data generated by HPLC-MS/MS instrument containing both standard and HCM samples were manually processed using the Quanbrowser module of Xcalibur 4.0, to be converted into relative abundance in Microsoft Excel. Peak area data were filtered if coefficient variant (CV) is below 0.25 from QC and normalized with bacterial plate count from daily HCM plating. The relative abundance of each SCFA (acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, hexanoic acid, isocaproic acid, n-heptanoic acid and 2-methylbutyric acid) was calculated using a calibration curve established from standards prepared. For polar metabolites, the raw data from LC/MS was processed using Compound Discover 3.1 and were filtered (CV < 0.25) and normalized before it was imported to MetaboAnalyst for further analysis. PLSDA plots and VIP score plots were generated to show the differences in the relationship between the pre-treatment and treatment phase. The above process was repeated for the processing of phenolic compounds.

Results

In this thesis study, we aim to uncover how a representative dietary component, black raspberry, can influence human gut microbial composition and metabolism, so we can eventually understand its gut microbiota-mediated health effects on humans. We hypothesize that the addition of BRB as a dietary intervention will remodel microbial metabolism and alter the production of potentially beneficial metabolites such as straight-chain short-chain fatty acids (SCFAs). The HCM contains three vessels representing ascending, transverse and descending colons, each vessel has temperature and pH sensors for environmental control and auto-pumps for feeding delivery at fixed time intervals. The HCM was run for 6 weeks to observe the longitudinal change in microbiota and metabolites produced when BRB was added as treatment. Representative samples were then selected from the collection of samples based on t-test significance using daily bacterial culture plate count and undergo further metabolomics analysis. The metabolomics study was performed to detect the changes in metabolites content between the pre-black raspberry treatment phase, black raspberry (BRB) treatment phase and post-black raspberry treatment (washout) phase.

Distinct Metabolic Profiles of Polar Metabolites Can Be Detected in Response to BRB Intervention in Different Section of the Colon

Polar metabolites are a group of small, hydrophilic molecules such as amino acids, nucleic acids, small organic acids, which are often involved in primary metabolism [24]. They are critical in normal growth and are crucial for survival in living organisms such as

microbes. In order to find out how polar metabolite production will respond to BRB intervention, we did a target polar metabolites analysis and hypothesize that the production of polar metabolites increases as more microbes grow with BRB.

In this study, after filtering and normalizing the data obtained, there were a total of 245 metabolites included in the analysis. Upon completion of the analysis, all three sections of the colon had shown distinct endogenous polar metabolic profiles when comparing the pre-treatment phase to the treatment phase. PLSDA plot of ascending colon had shown the distinct polar metabolites profile by comparing pre-treatment (in pink) to treatment phase (in green) (Figure 2A). For ascending colon, of the ten metabolites that contributed to the distinct metabolic profile, myristic acid contributed the most to the distinct metabolite profile observed (Figure 2B). Just like ascending colon, distinct polar metabolites profile was observed in the transverse colon as shown by PLSDA plot by comparing pre-treatment (in pink) to treatment phase (in green) (Figure 2C). For transverse colon, of the ten metabolites that contributed to the distinct metabolic profile, (5E,7E)-9,10-Secocholesta-5,7,10-triene-3,25-diol contributed the most to the distinct metabolite profile observed (Figure 2D). A similar trend is observed in descending colon as it also had a unique metabolic profile when comparing pre-treatment to treatment phase (Figure 2E). For transverse and descending colon, they had shared most of the metabolites that contributed to the diverse metabolic profile, such as linoleic acid and 9-hydroperoxy-10E,12Z-octadecadienoic acid (9-HpODE).

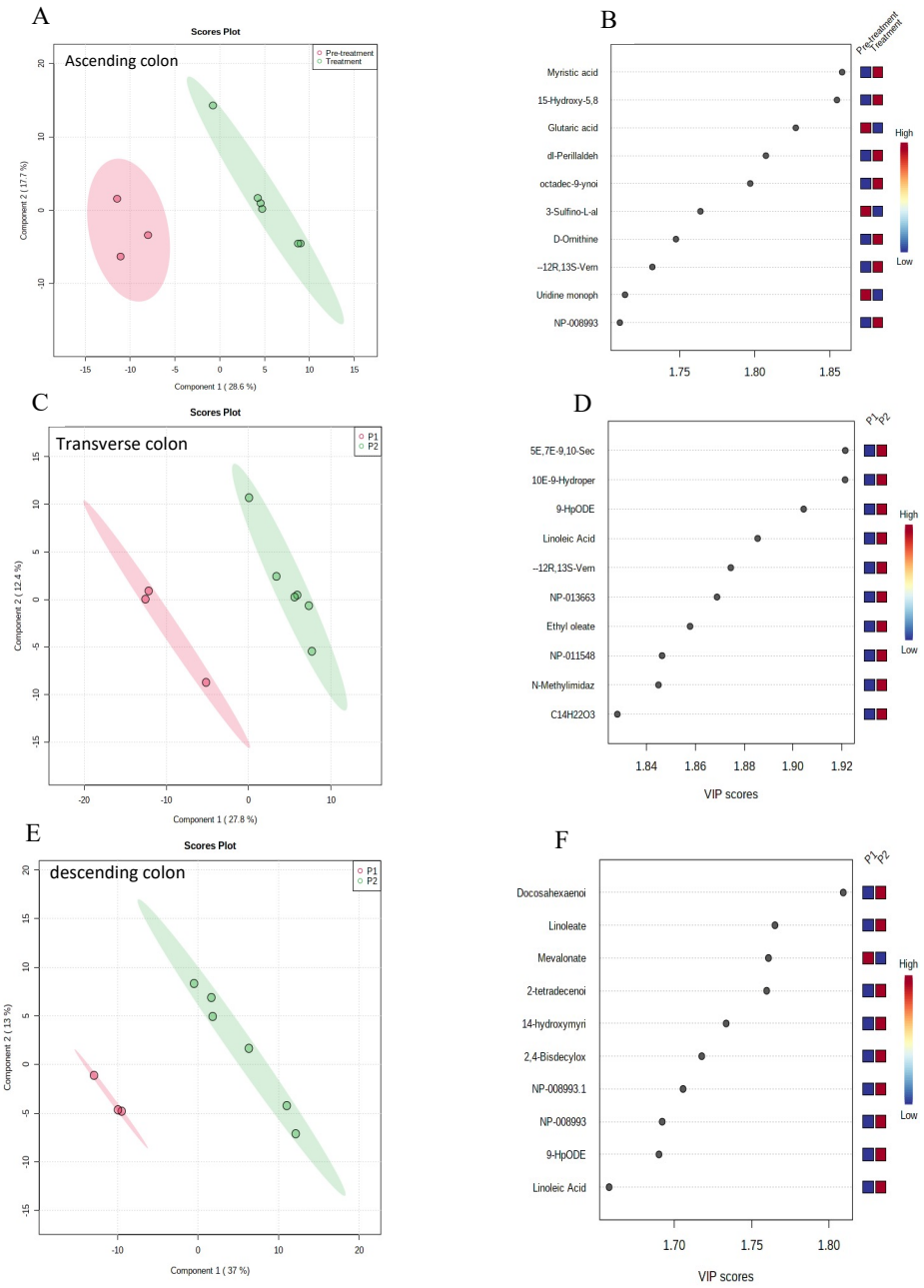

Figure 2: the panel of figures shown the polar metabolite changes in different vessels representing ascending colon, transverse colon and descending colon. **A, C, E:** PLSDA plot showing the distinct profile present in specific section of the colon by comparing pre-treatment (in pink) to treatment phase (in green). VIP scores plot shows the top 10

metabolites that contributed the most to the distinct metabolic profiles between the two phases of the experiment in **B:** ascending colon, **D:** transverse colon **E:** descending colon and the list was derived using MetaboAnalyst algorithm using peak area.

To compare how different sections of the colon respond to BRB treatment, a comparison of HCM vessels was performed to reveal the different metabolite profiles during the treatment stage in all three sections of the colon. All three sections of the colon had shown distinct changes in the metabolic profile during the treatment phase, which was mainly driven by the abundance of 14-hydroxymyristic acid, uridine monophosphate and trans-cinnamic acid (Figure 3A-B). It is also worth noting that the trend of metabolite relative abundance changes as shown in the heatmap in ascending colon was unique compared to the later sections of the colon while transverse and descending colon share a similar trend in how metabolic relative abundance changes (Figure 3C). For example, for M1 to M7, ascending colon is showing a decreasing trend in the production of these polar metabolites while transverse and descending colons were showing an increasing trend in the production of these metabolites.

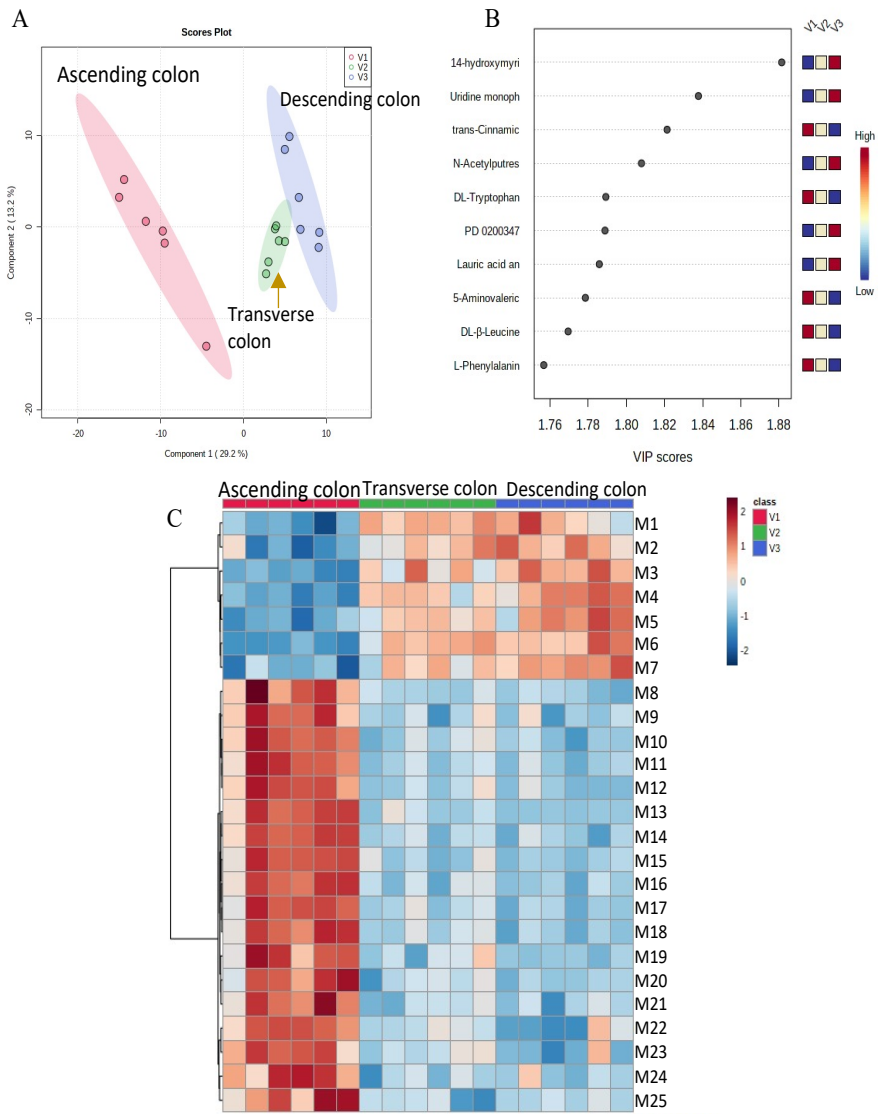

Figure 3: localizing comparison of three sections of the colon while black raspberry extract was added as the treatment and its impact on endogenous polar metabolic profile of microbiome in HCM. **A:** PSLDA plot of showing the distinct profile present in ascending (in pink), transverse (in green) and descending (in blue) colon during the treatment phase. **B:** VIP scores plot of the top 10 metabolites that were contributing to the distinct metabolic profiles between the two phases of experiment in HCM. **C:** heatmap showing the trend of top 25 metabolites responded to BRB extract while HCM was running.

Significant Changes Can Be Detected in Phenolic Compounds between Treatment Versus Wash Out Phases

Polyphenol is a group of secondary metabolites that are commonly found in fruits, vegetables and plant-derived food such as chocolates. It was reported in previous studies that polyphenol can regulate microbial composition balance and enhance the growth of beneficial metabolites through their metabolites and thus behave like prebiotics [25]. Polyphenols can be dived into flavonoids or non- flavonoids and BRB extract contains both of these groups [18][26]. In order to understand how polyphenol can correct dysbiosis and enhance the production of beneficial compounds, we performed targeted profiling for polyphenol and its derivatives in metabolism. We had shortlisted 30 compounds for target phenolic compounds analysis. Out of 30 compounds in the Mzvault database, 12 were detected in BRB extract only, 5 compounds were detected in ascending colon of HCM. Of the 5 compounds, 2 of them, namely protocatechuic acid and hesperetin, had shown a significant increase in the peak area detected during the treatment phase and post-treatment phase, with t-test $p< 0.005$ and $p<0.001$ respectively (Figure 4A). For transverse colon, similar in the number of metabolites detected, quercetin and naringenin had shown a significance in the relative abundance of metabolites detected. Quercetin had shown a significant decrease in peak area during the treatment phase and washout phase, while naringenin was significantly increased, treatment phase and washout phase, with t-test $p< 0.001$ and $p<0.05$ respectively (Figure 4C). There is no significant change reported in descending colon, which might be due to the dilution effect in the vessels and thus the phenolic compounds were too diluted to be detected by microbes to cause significant changes.

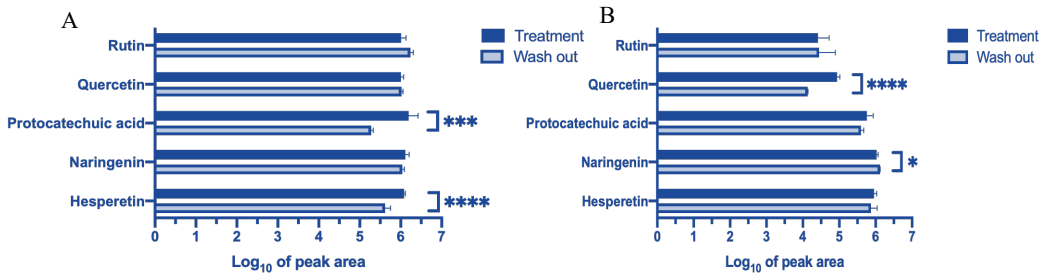

Figure 4: panel of figures showing phenolic metabolites analysis. **A:** figure showing five metabolites that were detected in ascending colon of HCM. Of the five metabolites, protocatechuic acid and hesperetin had shown significance change in the amount of metabolites detected during treatment phase and post treatment phase, with t test $p< 0.005$ and $p<0.001$ respectively **B:** figure showing five metabolites that were detected in ascending colon of HCM. Of the five metabolites, quercetin and naringenin had shown significance change in the amount of metabolites detected during treatment phase and post treatment phase, with t test $p< 0.001$ and $p<0.05$ respectively. Figures: courtesy of Shiqi Zhang.

BRB Extract Can Positively Impact on Production of Short-Chain Fatty Acids in Ascending and Transverse Colons

Short-chain fatty acids (SCFAs) are products of anaerobic fermentation by microbes in the large intestine [27]. Microbes, such as *Bifidobacteria* or *Lactobacilli*, can act on polysaccharides that were not used by humans such as dietary fiber and resistant starch [28]. SCFAs can be dived into 2 groups: branched and unbranched and we had decided to focus on both of these groups as each will impact human health differently and produced by a different group of bacteria. If the amount of probiotics increases, linear SCFA increases. In our study, there was 9 total short-chain fatty acid included for target profiling (acetic acid, propionic acid, isobutyric acid, butyric acid, 2-methylbutyric acid, isovaleric acid, valeric acid, isocaprioic acid and hexanoic acid). In ascending colon, all 9 SCFAs were detected but 6 of them were detected at higher abundance. We were unable to quantify the amount of acetic acid present in the ascending colon as its peak area did not fit the range of the calibration curve. Statistical analysis using t-test had shown that propionic acid, isobutyric acid, 2-methylbutyric acid and hexanoic acid, had shown a significant increase in the concentration between pre-treatment to treatment phase in ascending colon (t-test $p < 0.05$) (Figure 5A). Valeric acid had also shown a significant increase in the concentration between pre-treatment to treatment phase in ascending colon (t-test $p < 0.05$). Isovaleric acid had shown a significant increase in the concentration between pre-treatment to wash out phase with t-test $p < 0.01$ (Figure 5A). With regards to the transverse colon, 7 metabolites had been detected and 2 of them, namely propanoic acid and isovaleric acid, had shown a significant increase between pre-treatment to wash out

phase (t-test p< 0.05) (Figure 5B). There were no significant changes detected in the abundance of SCFA in descending section of the HCM (data not shown).

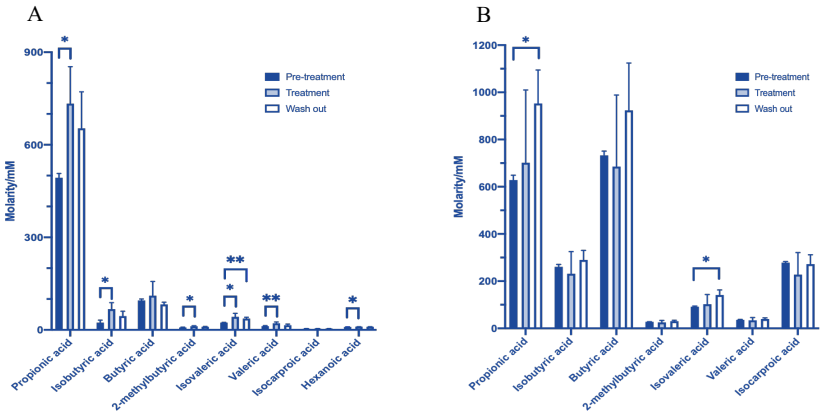

Figure 5: **A:** figure showing short chain fatty acid relative abundance in ascending colon, 6 out of 9 metabolites had shown significant changes between the three phases of experiments. Statistical analysis shown that propionic acid, isobutyric acid, 2-methylbutyric acid and hexanoic acid shown a significant increase in concentration between pre-treatment to treatment phase in ascending colon (t-test p <0.05). Isovaleric acid and valeric acid also shown a significant increase in their concentration (t-test p <0.01). there are no significant changes detected in other sections of the colon. **B:** figure showing short chain fatty acid relative abundance in transverse colon, 2 out of 9 metabolites had shown significant changes between the three phases of experiments. Statistical analysis shown that propionic acid, isovaleric acid shown a significant increase in concentration between pre-treatment to treatment phase in ascending colon (t-test p <0.05). There is no significant changes detected in descending colon and thus figure was not shown. Figures: courtesy of Shiqi Zhang.

Discussion

The original intent of this thesis study was aimed to uncover how a representative dietary component, black raspberry (BRB), can influence human gut microbial composition and metabolism, so we can understand its gut microbiota-mediated health effects on humans. However, due to pandemic impact, metagenomic processing is still ongoing and thus, we now focus on how BRB can impact the microbial metabolism and the production of its associated metabolites (polar, phenolic and short-chain fatty acids), and try to establish a foundation for the study of their future impact to human health. We hypothesize the dietary intervention of BRB will remodel gut microbial metabolism and alter the production of potentially beneficial metabolites. In order to establish the link on how BRB could remodel microbial metabolism, I had utilized an innovative HCM model to mimic the human intestine environment without host interaction to study explicitly the BRB-gut microbiota interactions. The HCM was run for 6 weeks to allow the observation of longitudinal changes in microbiota and their metabolites, univariate and multivariate data analysis approaches were also applied to draw scientific conclusions.

Potential Physiological Benefits of Our Detected Microbial Polar Metabolites and their Roles in Cancer Prevention/Treatment

From the polar metabolites results, we can see that BRB extract is capable of inducing metabolic changes in all three sections of the colon for endogenous polar metabolites produced by gut microbes, with stronger similarity in the metabolic changes between transverse and descending colons. Polar metabolites are a group of small,

hydrophilic metabolites such as amino acids, nucleic acids, sugars and other organic acids that plays a vital role in normal growth and development in living organisms, as they are typically part of the primary metabolism [24]. Changes in endogenous polar metabolites thus signify there are changes in microbial growth and potentially microbial diversity. In this experiment, both 9-HpODE and linoleic acid were increased in the transverse and descending colons. 9-HpODE is formed from the non-enzymatic oxidation of linoleic acid [29]. It is reported that in a cohort study, where comparison was made on the serum metabolites between healthy volunteer and colorectal cancer patient, they found that linoleic acid can serve as a biomarker for colorectal cancer as its relative abundance is much lower in colorectal cancer patient as compared to healthy volunteer [30]. In a mouse study on the intestinal tumor, researchers also found that linoleic acid, a long-chain fatty acid, has an anti-tumor effect on specific cell lines of cancer such as KPL-1 and BT-474 in breast cancer, Caco-2 in colorectal cancer, A549 in lung cancer and DU145 in prostate cancer [31]. Briefly, an increased intake of n-3 polyunsaturated fatty acids from food, such as linoleic acid, was able to reduce the risk for breast cancer development [32][33]. Such a result was confirmed as in a reported animal study that prostaglandin E (PGE) production decreases when female BALB/c mice were fed with linseed oil, decreasing PGE reduces the growth of tumor [34]. Since BRB extract can upregulate the production of linoleic acid, BRB dietary intervention might have a similar effect on other cancer patients as PGE is found universally in many cancers, including lung cancer and its concentration is directly proportional to the growth rate of lung cancer [35].

It was reported in a literature review that different locations may have different microbial populations thus, contribute to different colon cancer incidence [36]. After

comparing three sections of the colon via location analysis, I discovered that ascending colon has a unique trend while the transverse colon and descending shared a similar trend in endogenous polar metabolites production. As BRB treatment feeding medium first enter the ascending colon (Vessel 1), the microbes were exposed to 5ml/L of BRB extract. However, the concentration of fresh BRB extract will become much lower in the transverse and descending colon as the media were first utilized by microbes in ascending colon and was diluted by a great extent when passed on to following colon compartments. As such, the remaining BRB extract exposed to transverse and descending colons might not meet the concentration thresholds to create a similar change to ascending colon. Therefore, I hypothesis that transverse and descending colon will have a similar metabolic profile as they are exposed to a similar concentration of BRB extract while ascending colon received fresh media. Location analysis also provided insights into the important metabolites that contributed to the distinct metabolic profile. In my experiment, the uniqueness of metabolic profile in a different section of the colon was contributed by the differences in concentration present in 14-hydroxymyristic acid, uridine monophosphate and trans-cinnamic acid across all sections of the colon. Of the three metabolites, trans-cinnamic acid is able to induce cancer cell death by the inhibition of histone deacetylases in athymic mice of colon cancer [37]. Similar findings had also been reported for cervical cancer via the same mechanism [38]. With all these information, I hypothesis that an increase in trans-cinnamic acid could curb the proliferation in other cancer patients too.

Potential Physiological Benefits of Detected Phenolic Compounds from the Gut Environment

Phenolic compounds are a group of bioactive ingredient that has antioxidant and cytotoxic activities [16]. Like many other berries, such as strawberry and pomegranate, black raspberry contains a rich source of anthocyanins and poorly absorbed ellagitannins [16]. In this study, we had observed that 2 out of 5 phenolics detected have statistical significance in selected sections of the colon, namely protocatechuic acid and hesperetin for ascending colon and quercetin and naringenin in the transverse colon. Protocatechuic acid, hesperetin and quercetin had decreased drastically when BRB extract was removed during the washout phase, indicates that the decrease was from withdrawing effects. Such observation is consistent with a previous study done for quercetin in a mice model using lychee pulp [39]. However, naringenin increased after BRB extract was removed, suggesting there might be microbes that are capable of producing such phenolic compounds from other phenolic components in BRB. As reported by Chen *et al*, both naringenin and 3-(4′-hydroxyphenyl) propanoic acid (HPPA) are products of naringin [40]. Therefore, the increase in naringenin might be attributed to the increase in naringin present in the HCM and naringin is derived from rutin [41]. As there is a slight increase in rutin in ascending colon, the excess might be transferred over to the transverse colon and resulted in such a significant increase. There is no statistical significance observed for polyphenols in descending colon as the BRB media might be too diluted after passing through the ascending and transverse section of the colon.

Existing literature has discussed the biological function of polyphenols in humans. For example, protocatechuic acid was known to have antibacterial, anticancer and anti-inflammatory activities [11]. It was reported by Suzuki *et al* that using the male F344 rats model, protocatechuic acid exhibited chemo-preventative activity as it can decrease the

likelihood of precancerous lesions and metastasizing to the lungs [42]. Chemo-preventative effects were also seen in hesperetin, quercetin and naringenin *in vitro* studies for breast cancer (MDA-MB-435 and HER2) [43][44]. Specifically, in HER2-positive cancer, hesperetin and naringenin can promote cancer cell death by functioning as a HER2 tyrosine kinase inhibitor [44]. Taken together, our limited results of phenolic compounds may suggest their involvement in cancer prevention, development and treatment in certain tumors. However, more mechanistic studies have to be performed down the road using these phenolic compounds to truly allow the discovery of their biological functions in modulating gut microbial metabolism and consequentially impact human health.

Potential Physiological Functions of Short-Chain Fatty Acids

Short-chain fatty acids (SCFAs) are defined as any fatty acids less than six carbons, most of them were the product of anaerobic fermentation of fibers from the diet, while the rest were produced from the lactate pathway [45]. In this study, we had included nine SCFAs in our analysis, namely acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocaproic acid and hexanoic acid. Most of which are straight-chain fatty acids, except for isobutyric acid, isovaleric acid and isocaproic acid. It was reported in many publications that an increase in many SCFAs has health benefits, including but not limited to anti-cancer effects, decreasing the production of proinflammatory cytokines, alter gut integrity and weight loss [46][47][48]. There are SCFAs detrimental health impacts as well, where high levels of branched SCFAs in the fecal sample correlate with obesity, a risk factor for many chronic diseases [49].

In our study, we had observed that 6 out of 9 SCFAs (propionic acid, isobutyric acid, 2-methylbutyric acid and hexanoic acid, isovaleric acid and valeric acid) were statistically significant between the pre-treatment and treatment phase in ascending colon. The changes of our detected propionic acid and valeric acid are consistent with a mice study done by Huang *et al* using lychee pulp [50]. It was reported that propionate and butyrate were important metabolites to maintain gut homeostasis, where people with active inflammatory bowel disease (IBD) contain less of these SCFAs in their fecal sample as compared to healthy adults, and IBD is an indication of dysbiosis [51]. It was also reported that prebiotics can improve such dysbiosis [25]. As the SCFA concentration increases in our study after BRB extract was added, this might be an indication that polyphenol in BRB can improve the microbial metabolic profile.

Many researchers also looked into the anti-cancer effects of SCFAs using *in vitro* studies or mice models [52]. Butyric acid was well studied in its mechanism in cancer prevention and treatment. Kuefer *et al* did an in vitro study on prostate cancer and found that sodium butyrate would acetylate p53 that induces p21, which then inhibits the activity of cyclin-dependent kinase 2. The result of such cellular action is cell cycle is arrested in the G1 phase of the cell cycle [53]. Work done by Terui's group further shows that p53 activation upregulates PIG3 and NOXA associated pathways, leading to cell death [54]. Another aspect of anti-cancer effects of butyrate is to activate the death receptor pathway components, which was demonstrated in the work done by Hernandez *et al* and Kim *et al*. *In vitro* study had shown that sodium butyrate increases the sensitivity of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) resistant colon cells to undergo apoptosis [55]. Also demonstrated using colon cancer cells, sodium butyrate can upregulate

expression of DR5, a membrane of TRAIL, so to initiate cell apoptosis [56]. In our study, butyric acid increases between pre-treatment and washout phases in the transverse colon. This means that patients who have higher risk of developing lung cancer can benefit from the production of SCFAs in the host gut and thus, escalate host-mediated anti-tumor responses to curb the progression of cancer.

There were also studies done on the association of bacteria phylum on the production of SCFA metabolites. For example, a report by Derrien *et al*. suggested that probiotics like *Akkermansia muciniphila* are a major propionate producer [57]. Bacteria phyla like *Bacteroidetes*, also produce propionate, while butyrate was mainly produced by *Firmicutes* [45]. Both phyla are part of the commensal bacteria in the gut and account for in total 90% of the gut microbiota in humans [58]. Since there was an increase in the production of propionic acid in our study, we can extrapolate that such an increase in SCFAs might be due to an increase in certain probiotics after the addition of BRB. Such a hypothesis will be further confirmed by the result from metagenomic analysis in the future.

Conclusion

In summary, we explored the association between BRB intervention and gut microbial metabolism in this study, and discussed its potential health implication on the cancer patient. Our results show that BRB extract is capable of modulating microbiome metabolism as important groups of metabolites, namely polar, phenolic and short-chain fatty acids, had shown significant changes among pre-treatment, treatment and washout phases. Our data may be used to explain how BRB extract can function as prebiotics and promote the growth of probiotics, thus enhance the production of functioning metabolites that played a positive role in host physiological health. The increased production of beneficial metabolites, such as linear short-chain fatty acids, by gut microbes, can potentially curb tumor proliferation in cancer patients by downregulating the production of prostaglandin E, which is an important cancer growth factor that promotes cancer proliferation [59].

Although we speculate the increase in SCFAs may be due to the changes in the population of certain SCFA-producing bacteria, due to the incomplete process of metagenomics data collection impacted by COVID, we were unable to establish the link between microbial population and its metabolites production precisely so that we can pinpoint the significant change in polar and phenolic compounds to a specific species of bacteria. To complete the puzzle, what we would like to do next is to continue with the metagenomic study of our samples. Once the result from 16s rRNA and transcriptomics were out, as done previously in our lab, the Kyoto Encyclopaedia of Genes and Genomes database coupled with Statistical Analysis of Metagenomics profiles (STAMP) will be used

to analyze functional data [21]. This is to relate the changes in metabolomics study to microbial population and provide insight on how BRB extract might be able to correct dysbiosis and improve beneficial metabolites production through promoting the growth of probiotics.

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Supplementary table

Code	Full name/code of metabolites
M1	Pyridoxial
M2	GPK
M3	Uridine monophosphate
M4	14-hydroxymyristic acid
M5	Lauric acid anion
M6	PD 0200347
M7	N-Acetylputrescine
M8	D-Ornithine
M9	L-Alanine
M10	5-Aminovaleric acid
M11	L-Proline
M12	D-Proline
M13	Trans-Cinnamic acid
M14	L-Phenylalanine
M15	L-Methionine
M16	L-Tyrosine
M17	DL- beta-Leucine
M18	DL-Typtophan
M19	L-Alanyl-L-proline
M20	Glycyl-L-leucine
M21	Trans-3-Indoleacrylic acid
M22	Glycocholic acid H
M23	Glycocholic acid
M24	Leucine
M25	5Z,8Z,11Z,14Z-5,8,11,14-Tetraenoic acid